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The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus

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The argininosuccinate lyase (ASL) gene of *Chlamydomonas reinhardtii* has been cloned using four oligonucleotide probes corresponding to highly conserved regions of the ASL polypeptide sequence. The identity of the gene was confirmed by partial sequencing. It is unique, contains several introns and spans a region <7.8 kb that includes highly repetitive sequences. Using a particle gun, a reliable nuclear transformation system has been established by complementing three mutants deficient in ASL activity with the wild-type ASL gene. Analysis of the transformants reveals variable patterns of integration of the transforming DNA into the nuclear genome. Previous work has mapped the mutations in the mutants arg2 and arg7 to either end of the ARG7 locus 1.0 to 1.6 recombination map units apart. Our transformation results show that these two mutations are located within a region of 7.8 kb. This allows for the first correlation of the recombination map and the molecular map at the ARG7 locus and indicates a high recombination frequency in this region of the nuclear genome.

Key words: biolistic transformation/argininosuccinate lyase gene/recombination/repetitive DNA/*Chlamydomonas reinhardtii*/arginine auxotrophs

Introduction

In recent years the green unicellular alga *Chlamydomonas reinhardtii* has proven to be an attractive system for the study of several important biological problems such as photosynthesis, chloroplast biogenesis, flagellar biosynthesis, cell recognition and cell cycle regulation. One major problem, however, has been the lack of a reliable and efficient transformation system. Nuclear transformation of *C.reinhardtii* has been reported with two different selection methods. The first used arginine auxotrophs affected at the ARG7 locus which encodes argininosuccinate lyase (ASL), the last enzyme of the arginine biosynthetic pathway that converts argininosuccinate into arginine and fumarate. Transformation was achieved with the corresponding ASL gene of yeast (Rochaix and van Dillewijn, 1982; Rochaix *et al.*, 1984; Rochaix, 1987a). The second method used the sensitivity of *C.reinhardtii* cells to kanamycin. Transformation with the bacterial neophosphotransferase II gene fused to bacterial or eukaryotic promoters was reported to confer kanamycin resistance to transformed cells (Cox *et al.*, 1985; Hasnain *et al.*, 1985). However, neither of these selective markers allows for a high transformation efficiency and for reproducible results. Whether these problems are due to the poor expression of heterologous genes in *C.reinhardtii* or to the limited DNA delivery to the cells and/or the nucleus with the transformation procedure used is not clear.

We have developed a reliable nuclear transformation system in *C.reinhardtii* by making two major changes in the earlier procedures. First, an authentic *C.reinhardtii* gene has been chosen as the selective marker. We have cloned the ASL gene of *C.reinhardtii* because the corresponding locus, ARG7, has been extensively characterized genetically (Loppes and Matagne, 1972; Matagne, 1978) and several arg7 mutations are available that revert only at a low frequency (Gillham, 1965; Matagne, 1978). Second, we have constructed a particle gun and used it for delivering DNA to the cells (Klein *et al.*, 1987; Sanford *et al.*, 1987). This biolistic transformation method has already been used successfully for chloroplast transformation in *C.reinhardtii* (Boynton *et al.*, 1988).

The ARG7 locus is of special interest because a detailed genetic map has been established (Matagne, 1978). Here we show that the arg2 and arg7 mutations that have been mapped at both ends of the ARG7 locus, 1.0–1.6 recombination units apart, are both located within a 7.8 kb fragment that contains all the information for ASL expression. This represents the first correlation between the recombination map and the physical map of the ARG7 locus and indicates a high frequency of recombination which may be due to the presence of repetitive sequences both within the ASL gene and in its flanking regions.

Results

Cloning of the argininosuccinate lyase gene of C.reinhardtii

The sequences of the ASL genes of *Escherichia coli* (partial sequence, Charlier *et al.*, 1982), yeast (Beacham *et al.*, 1984), humans (O'Brien *et al.*, 1986; Matuo *et al.*, 1988) and rat (Amaya *et al.*, 1988) have recently been determined. We have taken advantage of the homology of the amino acid sequences of ASL in these different species to construct four oligonucleotides corresponding to the four most conserved regions (Table I). These probes called arg-mer, ASLI, ASLII and ASLIII were designed by taking into account the highly biased codon usage of nuclear genes of *C.reinhardtii* (Rochaix, 1987b).

Approximately 10^5 phage from a λgt10 cDNA library were screened with the four oligonucleotide probes. Although 50 phage hybridizing to the oligonucleotides were detected in the primary screen, no overlap was observed between hybridization patterns obtained with different oligonucleotides. Some inserts from responding phage were

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Table I. Oligonucleotide probes used to screen the λEMBL 3 library of *C.reinhardtii*

Probe
Arg-mer (corresponds to Lys_7-Thr_{14} of yeast ASL) GGT GAA GCG (GA)CC (GA)CC CCA CAG
ASLI (corresponding to $Asn_{84}-Thr_{90}$ of yeast ASL) GT GTG (TG)AT GTC CTC GTC GTT
ASLII (corresponds to $Met_{256}-Asn_{261}$ of yeast ASL) GTT CTT CTT CTG GGG CAT
ASLIII (corresponds to $Tyr_{378}-Gly_{390}$ of yeast ASL) (GA)CC CTT GCG (GC)AC CAG GTA

The oligonucleotides correspond to the strand complementary to mRNA. They were designed assuming the biased codon usage found for several nuclear genes (Rochaix, 1987b). Underlined bases correspond to mismatches.

sequenced and indeed shown to contain short sequences related to the oligonucleotides. However, mismatches and upstream and downstream sequences clearly indicated that these inserts were unrelated to the ASL gene. A new screening with 10^6 phage from the same cDNA library and from an independently constructed cDNA library did not provide any phage hybridizing to the four oligonucleotides. Screening was subsequently performed on 10^5 phage from a λEMBL3 genomic library (Goldschmidt-Clermont, 1986) with the four oligonucleotides. Arg-mer, ASLI, ASLII and ASLIII hybridized to 50, 62, 12 and 4 phage respectively. A single overlap with the four probes was found. This phage, λARG5-1, was purified and shown to contain a 12.6 kb *Sau*3A partial digest as insert. Its restriction map is shown in Figure 1. Another phage, λARG 8-1, was isolated which hybridized to the ASLII and ASLIII oligonucleotides but not to the arg-mer and ASLI oligonucleotides. The insert of this phage was shown to overlap the right part of the insert of λARG 5-1 (Figure 1). Digestion of λARG 8-1 with *Bam*HI revealed that the right end of the 12.6 kb *Sau*3A partial digest does not correspond to a genomic *Bam*HI site. Hybridization of a *Bam*HI−*Kpn*I total DNA digest with an ASL gene-specific probe indicated that the left end of the 12.6 kb *Sau*3A partial digest corresponds to a genomic *Bam*HI site (data not shown).

Partial sequence of the ASL gene

To confirm that λARG5-1 contains the ASL gene of *C.reinhardtii*, regions hybridizing to the four oligonucleotide probes were subcloned and sequenced. Each region shown in Figure 1 corresponds to an exon of the ASL gene that is flanked by typical eukaryotic splice donor and acceptor sites that follow the GT/AG rule (not shown in Figure 1). The amino acid sequences of these exons are highly related to the homologous regions of yeast and rat ASL. The similarities between the peptides encoded by the arg-mer, ASLI, ASLII and ASLIII exons and their yeast counterparts are 58, 65, 60 and 83% respectively. Comparison of the authentic sequences with the oligonucleotides indicates that the arg-mer and ASLII oligonucleotides have one mismatch and the ASLI oligonucleotide has three mismatched bases because of a non conserved amino acid (Table I). Exon L codes for the 39 carboxy-terminal amino acids of the *C.reinhardtii* ASL. This part of the protein is less conserved and has a homology of only 38% with the corresponding regions of yeast and rat ASL. From the preliminary ASL gene sequence data we estimate that this gene contains at least 10 introns.

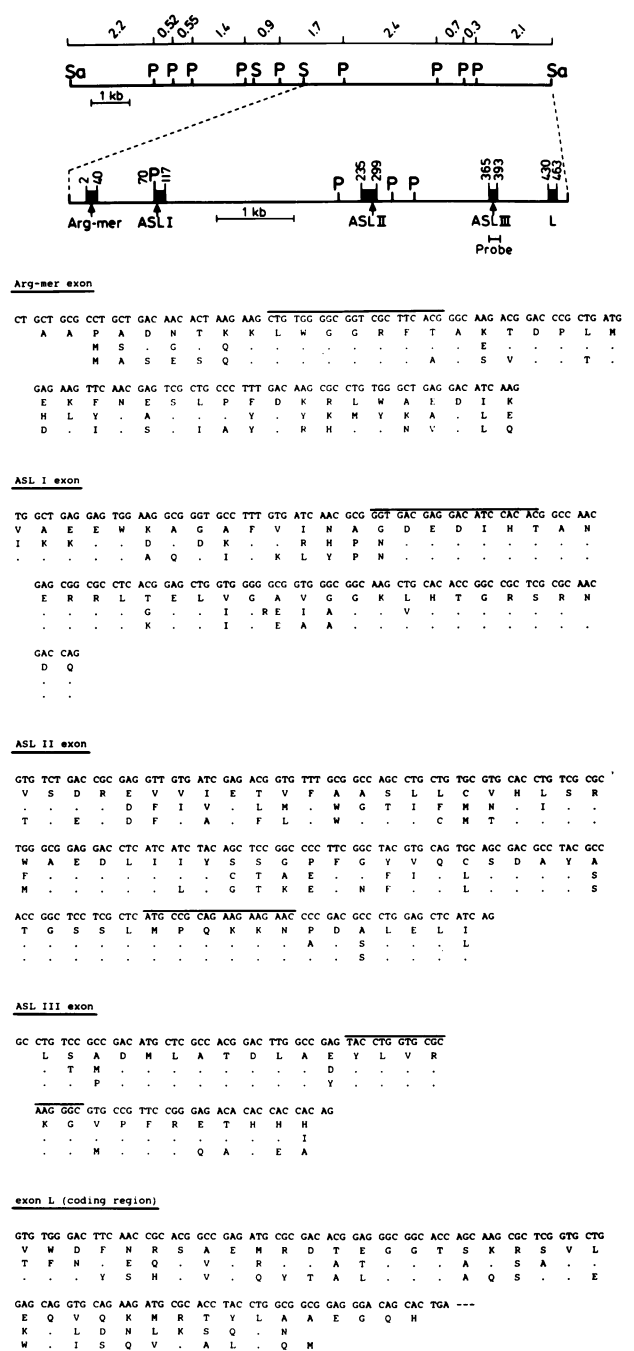

Fig. 1. Upper: restriction map of the 12.6 kb *Sau*3A partial digest containing the ASL gene from *C.reinhardtii*. Regions corresponding to the oligonucleotides arg-mer, ASLI, ASLII and ASLIII are indicated by arrows. The corresponding exons are marked with black bars. L designates the last ASL exon (only the coding region is shown). Numbers at both ends of each exon correspond to the amino acid residues in the corresponding yeast ASL sequence. Sa, *Sau*3A (only the terminal *Sau*3A sites are indicated); P, *Pst*I; S, *Sal*I. Note that the left, but not the right *Sau*3A site, corresponds to a genomic *Bam*HI site. **Lower**: partial sequence of the *C.reinhardtii* ASL gene. Nucleotide sequences of the five exons indicated on the restriction map are shown. Amino acids are indicated in the single-letter code and correspond from top to bottom to *C.reinhardtii*, yeast (Beacham *et al.*, 1984) and rat (Matsubasa *et al.*, 1989). Dots indicate identical amino acids. Regions complementary to the oligonucleotides are marked by a continuous line above the sequence.

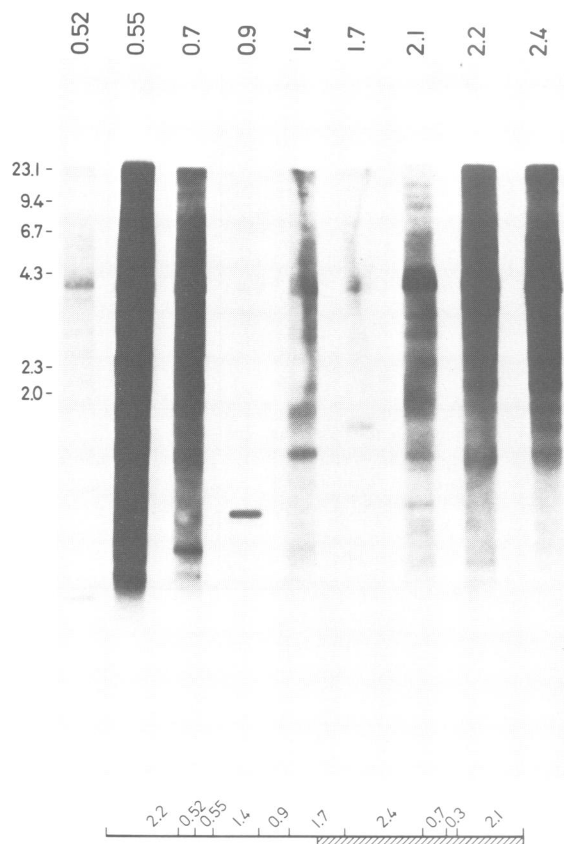

Fig. 2. The ASL gene region contains repeated sequences. DNA from arg7 cells was digested with *Pst*I, electrophoresed on agarose gels and hybridized by the method of Southern (1975) to each of the labelled *Pst*I fragments contained within the 12.6 kb ASL gene region which is shown at the bottom. The hatched bar indicates the ASL gene. After hybridization the filters were washed in 0.1 × SSPE, 0.1% SDS at 70°C for 1 h. The probes used for each lane are indicated at the top of the figure. Numbers on the left correspond to λ*Hin*dIII fragments used as size markers.

Repetitive sequences within and outside the ASL gene

Hybridization of the 12.6 kb insert of λARG5-1 with an *Eco*RI digest of total DNA from *C.reinhardtii* produced a strong smear (data not shown), indicating that the insert contains one or more repeated sequences. To localize these repeated sequences within the ASL gene region each *Pst*I fragment from the phage insert was isolated, labelled and hybridized with a *Pst*I digest of total *C.reinhardtii* DNA (Figure 2). It can be seen that several *Pst*I fragments from the λARG5-1 insert hybridize to highly repeated sequences. Two of these, the 2.4 and 0.7 kb fragments are entirely localized within the ASL gene. The presence of repeated sequences from the same family on different *Pst*I fragments of the phage insert was also tested by hybridization (data not shown). We found that the 2.4 kb *Pst*I fragment shares common sequences with the 0.7, 2.2 and 0.55 kb fragments. The latter two fragments are located upstream of the ASL gene (Figure 2).

Transformation of the C.reinhardtii arg7 mutant with the wild-type ASL gene

The ASL gene was used for the transformation experiments. The intact 12.6 kb insert of λARG5-1 was cloned as a *Bam*HI fragment in pUC19 to produce the recombinant

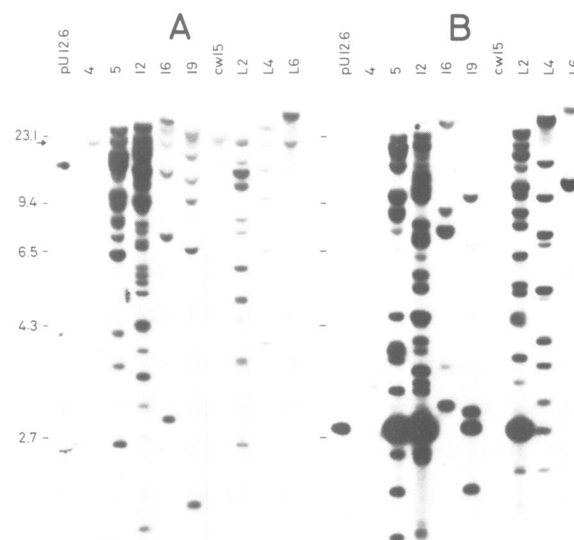

Fig. 3. Analysis of arg7 transformants. (**A**) DNA from the transformants 4, 5, 12, 16, 19, L2, L4 and L6 was digested with *Bam*HI, electrophoresed on a 0.8% agarose gel and hybridized with an ASLIII-specific probe (130 bp *Sph*I–*Stu*I fragment, cf. Figure 1). (**B**) After the hybridization shown in (A) the probe was eluted and the filter was rehybridized with labelled pCU19 DNA. Size markers indicated on the left are the same as those shown in Figure 2. Cw15 is an untransformed *C.reinhardtii* strain. The arrow marks the 17 kb *Bam*HI fragment containing the resident ASL gene.

plasmid pU12.6. Initially attempts were made to transform arg7 cells treated with autolysin or cell wall deficient arg7 cells using the previously established poly-L-ornithine method (Rochaix and van Dillewijn, 1982). The number of arginine prototrophs in these experiments was not significantly higher than in the control sample ($\sim 10^{-7}$). No attempt was made to analyse the DNA of these putative transformants. A significant improvement in transformation yield was obtained with a home-made particle gun (cf. Materials and methods). Plates containing $\sim 10^7$ arg7 cells were bombarded with the pU12.6 plasmid and after several days arginine prototrophs appeared at a frequency of 20–50 colonies/plate. Several colonies were grown and their DNA was isolated and examined by Southern hybridizations. Transformants 4, 5, 12, 16 and 19 were obtained with supercoiled pU12.6 plasmid, while transformants L2, L4 and L6 were obtained with pU12.6 digested with *Bam*HI which released the insert. No significant difference in transformation efficiency was observed with supercoiled and linear DNA.

Figure 3A shows the results obtained when DNA from the transformants was digested with *Bam*HI and hybridized with a 130 nucleotide *Sph*I–*Stu*I fragment containing the ASLIII region. A small probe lacking repetitive DNA was required in order to avoid cross-hybridizations with repeated sequences that are interspersed throughout the ASL gene region and the nuclear genome. This probe hybridizes with a unique *Bam*HI fragment of 17 kb in the untransformed strain cw15 (Figure 3A) and the arg7 mutant (data not shown). Hybridization of *Sau*3A digests of arg7 and cw15 DNA with the same probe reveals the presence of a unique 1.75 kb fragment (data not shown). These results indicate that the ASL gene is a single-copy gene in *C.reinhardtii* in agreement with genetic data (Gillham, 1965). The numerous hybridizing bands in the *Bam*HI digests of the transformants

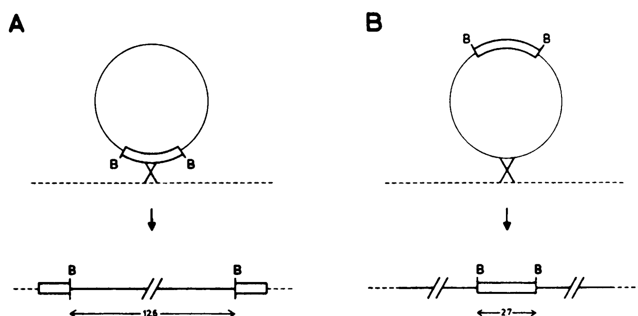

Fig. 4. Models for simple integration of the transforming plasmid pU12.6 into the nuclear genome. (**B**) Recombination occurs within the insert of pU12.6. The vector bounded by two *Bam*HI sites (B) is maintained intact upon integration. (**A**) Recombination occurs within the vector portion of pU12.6. The 12.6 kb insert bounded by two *Bam*HI sites is maintained intact upon integration. — — — nuclear DNA, —— insert of pU12.6, ▭ vector portion of pU12.6.

suggest that the pUC12.6 DNA has integrated in the nuclear genome at several sites. If the pU12.6 plasmid replicated autonomously in transformants one major signal in the *Bam*HI digest would be expected at 12.6 kb in Figure 3A corresponding to the free insert. The same hybridization signal would also be expected if the integration of the transforming plasmid occurred by recombination between the vector portion of the plasmid and nuclear DNA (cf. Figure 4A). Figure 3A shows instead that most of the bands have sizes ranging from <2 kb to >20 kb. This indicates that integration sites of the transforming plasmid occurred mostly through sites within the insert rather than in the vector portion (cf. Figure 4B). It is also possible that some of the transforming plasmid has been rearranged. Integration of the transforming DNA into the nuclear genome was confirmed by performing hybridizations with DNA digested with *Eco*RV which does not cut the pU12.6 plasmid. Comparison of hybridization patterns of *Eco*RV digested and undigested DNA with labelled pUC19 DNA revealed that the hybridizing bands were significantly smaller in the digested than in the undigested samples (data not shown).

Figure 3B shows the hybridization of the same filter as in Figure 3A with labelled pUC19. A large number of hybridizing fragments can be seen, especially in transformants 5, 12 and L2. As expected for a *Bam*HI digest, most of these bands do not co-migrate with those hybridizing to the ASLIII probe. The strong hybridizations observed at 2.7 kb in the tranformants 5, 12, 19 and L2 confirm that recombination occurred principally within the insert of pU12.6. However, several other bands that are larger or smaller than pUC19 are observed in the DNA of these transformants. These bands could be explained by recombination of pUC19 sequences with nuclear DNA. In this case one would predict the existence of a 12.6 kb fragment hybridizing with the ASL III probe (cf. Figure 4A). Since this is not observed (Figure 3A) one can conclude that rearrangements have occurred in the transforming plasmid upon integration. These rearrangements appear, however, to be limited because Southern hybridization with *Taq*I digests of the DNA from the transformants reveals that most of the major fragments correspond to internal pUC19 fragments (data not shown).

It is noteworthy that although transformant L2 was obtained with *Bam*HI-digested pUC12.6 DNA one still observes a major hybridization with the 2.7 kb fragment of pUC19. This can be explained by the formation of pUC19 oligomers in the transformants prior to integration into chromosomes. In this case it is likely that integration occurred through non-homologous recombination since no significant cross-hybridization is detectable between pUC19 and *C.reinhardtii* DNA. It can be seen that sequences homologous to pUC19 are present in all transformants obtained with *Bam*HI digested pU12.6. This high frequency of co-transformation was confirmed by analysing six other transformants all of which contain pUC19 sequences (data not shown).

The multiple bands in the *Bam*HI hybridization obtained with the ASLIII probe indicate that most integrations occur by non-homologous recombination. Homologous recombination would leave the 17 kb fragment containing the ASL gene intact. At first sight such a gene replacement may have occurred in transformant 4 whose unique band hybridizing to the ASLIII probe is undistinguishable from the resident ASL *Bam*HI fragment. However, hybridization of a *Hin*dIII digest of the DNA from this transformant with the same probe reveals two bands, one of which corresponds to the resident fragment containing the ASLIII region, whereas the other can be attributed to the transforming DNA (data not shown). It appears, therefore, that in this transformant the resident 17 kb *Bam*HI fragment co-migrates fortuitously with the new band generated by the transformation. An indication that no homologous recombination has occurred in trans-

Table II. Specific activity of ASL in the transformants

Wild-type	100	100
arg-7	0	0
4	87	79
5	87	70
12	238	288
16	50	51
19	79	63
L2	92	81
L4	110	125
L6	103	119
L10	86	88

Values from two independent experiments are expressed as a percentage of wild-type ASL specific activity (1.1 μmol arginine/mg protein/h). Numbers of the transformants correspond to those shown in Figure 3 except for L10 which is not shown in Figure 3.

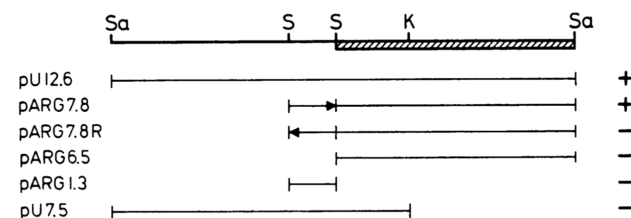

Fig. 5. Summary of transformations of arg7, arg7-3 and arg2 mutants obtained with plasmids containing different parts of the ASL gene region. Only the two *Sal*I (S) and the *Kpn*I (K) sites are shown on the 12.6 kb insert of pU12.6. The hatched bar indicates the ASL gene. In pARG7.8R the 1.3 kb *Sal*I fragment has been inverted relative to pARG7.8. + high transformation yield (10−50 colonies per plate); − transformation yield not significantly higher than reversion rate (0−1 colonies/plate).

formants 4 and L2 has been the recovery of arginine-requiring segregants from crosses between these transformants and wild-type (J.van Dillewijn and J.-D.Rochaix, unpublished results).

It is also possible that the pU12.6 plasmid can integrate by homologous recombination at any of the repeated sites in the nuclear genome to which pU12.6 cross-hybridizes (cf. Figure 2). Hybridization with the ASLIII probe would result in one or several bands of non-predictable size besides the resident 17 kb *Bam*HI fragment. Since a similar pattern of hybridization would result from non-homologous recombination it is not possible to distinguish between homologous and non-homologous recombination events in these transformants.

Expression of the ASL gene

Several attempts to detect the transcript of ASL by Northern hybridization with gene-specific probes failed both with total RNA and polyadenylated RNA from wild-type and from transformants. No discrete band was visible under conditions where the two transcripts from the genes encoding the small subunit of ribulose bisphosphate carboxylase/oxygenase were detected on the same filter after a short exposure. This result, together with our inability to isolate a cDNA clone from two independently constructed cDNA libraries, suggests that the ASL mRNA accumulates to low levels either because it is weakly transcribed or because it is unstable.

Measurements of ASL activity in several transformants are shown in Table II. In most transformants the activity is comparable to wild-type or slightly reduced. One exception is transformant 12 with an activity 2- to 3-fold higher than in wild-type. It can be seen in Figure 3A that both the ASLIII and the pUC19 probes hybridize to a large number of DNA fragments in this transformant.

Complementation of other mutations at the ARG7 locus by transformation

Studies on the ARG7 locus of *C.reinhardtii* have revealed the existence of mutations affecting ASL activity that can be grouped into several distinct complementation groups (Loppes and Matagne, 1972; Matagne, 1978). A genetic map of this locus has been established based on recombination frequencies which places the arg2 and arg7 mutation sites at either end of the locus and arg7-3 between arg2 and arg7. Genetic distances between arg2 and arg7-3, and arg7-3 and arg7 range between 0.2–0.6 and 0.8–1.0 map units respectively (Matagne, 1978).

To obtain an estimate of the physical distance between these genetic markers we have bombarded arg7, arg2 and arg7-3 mutants with plasmids containing various fragments of the ASL gene region. The results are summarized in Figure 5. It can be seen that all three mutants can be transformed with plasmids pU12.6 and pARG7.8. The latter contains a 7.8-kb DNA fragment which includes the ASL coding region and 1.5 kb of 5′ upstream region (cf. Figure 1). Attempts to transform the three mutants with smaller or rearranged fragments were mostly unsuccessful (see Figure 5) or yielded arginine auxotrophs at a frequency close to the reversion rate. We conclude that the arg2 and arg7 mutations are <7.8 kb apart on the chromosome. Since they are 1.0–1.6 recombination units apart on the genetic map a high rate of recombination occurs in this region.

Discussion

Isolation and identification of the ASL gene of C.reinhardtii

Four oligonucleotides corresponding to highly conserved regions of the ASL sequences from several organisms were used as probes for screening a genomic library of *C.reinhardtii*. A single phage, λARG5-1 was isolated and its 12.6 kb insert was shown to contain the ASL gene based on two independent criteria: partial sequencing and transformation of ASL-deficient mutants with this DNA.

Restriction fragments hybridizing with the four oligonucleotides were subcloned and partially sequenced. Each of the oligonucleotides belongs to a different exon coding for an amino acid sequence homologous to ASL peptides from yeast and rat (cf. Figure 1). Although the homology between the arg-mer exon and the yeast ASL sequence is apparent starting from the third amino acid of the yeast protein, no ATG initiation codon is present in this region. It is likely that an intron separates the initiation codon from the arg-mer region. An intron separating the fourth and fifth codon of the rat ASL gene has recently been found (Matsubasa *et al.*, 1989). Because the N-terminal end of ASL appears not to be highly conserved we have not been able to identify it with certainty in *C.reinhardtii*, although >700 bases have been sequenced upstream of the arg–mer region. It appears, however, that the 5′ end of the ASL gene and/or its promoter are included in the 1.3 kb *Sal*I fragment since arg7 mutants can be transformed with pARG7.8 but not with pARG6.5 (cf. Figure 5).

Nuclear transformation with the ASL gene as selective marker

The initial transformations of the arginine auxotrophs were performed with the plasmid pU12.6 which contains a 12.6 kb *Sau*3A partial digest isolated from a *C.reinhardtii* nuclear genomic library. Removal of 4.8 kb from this fragment produced a smaller plasmid pARG7.8 that transforms at the same frequency as pU12.6. From the partial ASL sequence and from the transformation results it appears that the promoter and the coding region of ASL are contained within the 7.8 kb insert of pARG7.8. Linearization of the plasmid did not significantly increase the transformation yield obtained with supercoiled plasmid. This is in marked contrast to yeast where the efficiency of transformation with integrating plasmids is increased 10- to 1000-fold by linearization (Orr-Weaver *et al.*, 1981).

Molecular analysis of several ASL transformants shows that in most cases the transforming DNA integrates at several sites in the genome. Genetic analysis of two transformants has indicated that the expressed copies of the introduced ASL gene are not linked to the ARG7 locus. This clearly proves that the insert in pU12.6 contains all the information needed to express ASL activity and that no integration by homologous gene replacement occurred at the ARG7 locus in these transformants. However, it is not possible to conclude that most integrations occur at non-homologous sites in these transformants because the DNA used contains sequences that are reiterated at numerous sites in the nuclear genome. It is therefore possible that homologous recombination occurs within these repeated sequences, i.e. homologous integrations may be driven outside the resident ASL gene region by the large number

of targets provided by the repeated elements. These events would lead to restriction patterns similar to those expected from non-homologous integrations.

Although most of the transformants appear to contain multiple inserts of the ASL gene, the specific activity of the enzyme is not increased relative to wild-type in most cases. It is known that regulation of arginine biosynthesis in *C.reinhardtii* occurs mainly at two steps, catalysed by *N*-acetylglutamate-5-phosphotransferase and ASL respectively. The first enzyme is inhibited by arginine (Farago and Dénes, 1967) and the activity of the second appears to be feedback regulated by some arginine product, perhaps arginyl-tRNA (Sussenbach and Strijkert, 1969). This feedback regulation may prevent a substantial increase of ASL activity in the transformants.

Relationship between recombination frequency and physical distance at the ARG7 locus

The ARG7 locus of *C.reinhardtii* has been studied extensively, especially since nearly half of all arginine-requiring mutants isolated lack ASL activity. Fine structure analysis has revealed that these mutants fall into five complementation groups and that the two outermost mutations of this locus, arg2 and arg7, are 1.0−1.6 recombination units apart (Matagne, 1978). One additional group includes mutants that are unable to recombine and to complement any of the other mutants at the ARG7 locus (Matagne, 1978). The availability of the ASL gene and of the nuclear transformation system has allowed us to correlate for the first time recombination map units with physical distance in the nuclear genome of *C.reinhardtii*. The transformations obtained with the arg2, arg7-3 and arg7 mutants indicate that these three mutations are all confined within a 7.8 kb nuclear DNA region. Since these mutants can complement each other (Matagne, 1978), the mutations are most likely localized within the coding region of ASL, which is ~6.5 kb long including introns. It therefore appears that 1.0−1.6 recombination units correspond to at most 6.5 kb of DNA at the ARG7 locus of *C.reinhardtii*. This recombination frequency is considerable, 20-fold higher than the average recombination frequency that can be estimated from the total map length and haploid DNA content of *C.reinhardtii* (~90−120 kb/cM; Harris, 1984). It is comparable to that found in yeast (4 kb/cM, Mortimer and Schild, 1984) and about two orders of magnitude higher than in *Drosophila* (~400 kb/cM; Treat-Clemons and Doane, 1984). There may be a link between the high recombination frequency observed and the repetitive sequences at the ARG7 locus. It remains to be seen how this frequency compares with that of other defined regions of the nuclear genome of *C.reinhardtii*.

The presence of repeated sequences within the ASL gene may explain the class of arg mutants that are unable to recombine with or to complement any of the other mutants at the ARG7 locus (Matagne, 1978). Since these mutations revert they cannot be deletions. Konvalinkova *et al.* (1974) suggest that they result from point mutations preventing the reading of the mRNA. We propose that these mutations result from DNA inversions either between repeated sequences within the ASL gene or between one repeated sequence in the ASL gene and another on the same chromosome. Mutants of this sort would be unable to complement any other mutants of the ARG7 locus because they produce an aberrant protein. Recombination between the inverted and normally oriented ASL gene would lead to lethal events and remain undetected.

Prospects

The *C.reinhardtii* ASL gene provides a reliable selective marker for transformation especially since the transformants can be selected on acetate-containing medium which allows for fast growth of the cells. The biolistic transformation procedure is simple and reproducible. We have introduced several foreign DNA fragments by co-transformation such as Ac, the autonomous transposable element from maize (Fedoroff *et al.*, 1983) and plasmids containing antibiotic markers driven by heterologous promoters (Day *et al.*, 1989). Attempts to express the neophosphotransferase II gene placed under the control of the SV40 promoter (Hasnain *et al.*, 1985) or the hygromycin resistance gene fused to the 35S promoter of cauliflower mosaic virus (Pietrzak *et al.*, 1986) were unsuccessful (A.Day and J.D.Rochaix, unpublished results). These preliminary results suggest a high specificity of *C.reinhardtii* promoters. There is little doubt that the ASL gene will be very useful as a selective marker in transformations aimed at complementating mutations that define regulatory genes involved in chloroplast biogenesis and flagellar assembly.

Materials and methods

Strains and media

Escherichia coli C600 Arg H^- was used as host strain for lambda libraries for screening with oligonucleotides. *Chlamydomonas reinhardtii* was grown on TAP medium (Gorman and Levine, 1965) supplemented when required with arginine (50 mg/l). The arginine auxotrophs arg7, arg7-3 and arg2 have been described (Gillham, 1965; Loppes and Matagne, 1972; Eversole, 1956).

Genetic analysis

Crosses of *C.reinhardtii* were performed as described by Levine and Ebersold (1980).

Oligonucleotide synthesis

The amino acid sequences of the argininosuccinate lyase of *E.coli* (Charlier *et al.*, 1982), yeast (Beacham *et al.*, 1984), human (O'Brien *et al.*, 1986) and rat (Amaya *et al.*, 1988) were compared. Four conserved peptides were chosen for the synthesis of oligonucleotides (cf. Table I). The nucleotide sequences were derived by using the biased codon usage found in nuclear genes of *C.reinhardtii* (Rochaix, 1987b).

Library screening

The *C.reinhardtii* cDNA λgt10 library and the genomic λEMBL 3 library were described previously (Goldschmidt-Clermont, 1986; Goldschmidt-Clermont and Rahire, 1986). A cDNA λgt11 library from *C.reinhardtii* gametes was kindly provided by S.Adair. Phage were plated on host *E.coli* C600 arg H^-. Each plate was replicated on four nitrocellulose filters. Phage lysis, DNA fixation and hybridization procedures were essentially as described by Maniatis *et al.* (1982). Oligonucleotides were labelled at their 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase. Each oligonucleotide was hybridized with one replica filter. The procedure of hybridization is described by Franzén *et al.* (1989). Positive recombinants were identified by overlapping signals obtained with at least two oligonucleotides.

DNA sequencing

Sequencing was performed on recombinant M13 by the chain termination method (Sanger *et al.*, 1977) with the modified T7 DNA polymerase (Tabor *et al.*, 1987) and the universal primer. The 1.25 kb *SalI*−*Sau*3A fragment hybridizing with arg-mer, the 2.3 kb *Sau*3A fragment hybridizing with ASLI, the 1.2 kb *Sau*3A fragment hybridizing with ASLII and the 1.5 kb *Sau*3A fragment hybridizing ASLIII were partially sequenced. Henikoff deletions (Henikoff, 1984) were used when the sequence corresponding to the custom oligonucleotide was not within the range of sequencing from the end of the insert.

Hybridization procedures

Southern blots were prepared and hybridized as described by Maniatis *et al.* (1982) except that BLOTTO (Johnson *et al.*, 1984) was used instead of Denhardt's solution. Northern blotting was performed according to Khandjian (1986). Probes were prepared using the 'random oligolabelling' technique (Feinberg and Vogelstein, 1983, 1984).

Transformation of C.reinhardtii

The particle gun constructed by our workshop was used according to the principle described by Klein *et al.* (1987). A detailed description of this gun will appear elsewhere. Transformation was performed essentially as described by Klein *et al.* (1987). Twenty-five microlitres of tungsten particle solution (100 mg/ml in 50% glycerol) were mixed with 2 μl DNA solution (1 mg/ml), 25 μl 2M $CaCl_2$ and 10 μl 0.1 M spermidine-base. The mixture was incubated 15 min in ice and centrifuged for 4 min in an Eppendorf centrifuge. After removal of 25 μl from the supernatant, the tungsten DNA pellet was resuspended in the remaining solution by brief sonication. Eight microlitres were layered on a nylon microprojectile that was carefully introduced into the barrel of the gun. Plates containing 10^7 cells on TAP-arginine agar medium were bombarded at a distance of 15 cm under vacuum (40 mbar residual pressure). After 1 day the cells were resuspended into 1 ml of TAP medium and evenly spread on 8-cm-diameter Petri dishes containing TAP agar medium and the cells were grown at 25°C under 2000 lux. Colonies appearing after one week were picked and grown in liquid TAP medium.

Argininosuccinate lyase activity

ASL activity was measured as described (Loppes *et al.*, 1972).

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